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Genetic Defects at the *UGT1* Locus Associated With Crigler-Najjar Type I Disease, Including a Prenatal Diagnosis

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Characterization of the *UGT1* gene complex locus encoding both multiple bilirubin and phenol UDP-glucuronosyltransferases (transferases) has been critical in identifying mutations in the bilirubin isoforms. This study utilizes this information to identify the bases of deficient bilirubin UDP-glucuronosyltransferase activity encoded by the UGT1A gene for the major bilirubin isozyme, HUG-Br1, in 3 Crigler-Najjar type I individuals and the genotype of an at-risk unborn sibling of one patient. A homozygous and heterozygous two-base mutation (CCC to CGT) created the HUG-Br1P387R mutant of the major bilirubin transferase in 2 different Crigler-Najjar type I patients, B.G. and G.D., respectively. Both parents of B.G. and his unborn sibling, J.G., were determined to be carriers of the P387R mutation. G.D. also contains the CAA to TAA nonsense mutation (G1n357st). Y.A. has a homozygous CT deletion in codons 40/41. The HUG-Br1P387R mutant protein was totally inactive at the major pH optimum (6.4), but retained 26% normal activity at the minor pH optimum (7.6), which was 5.4% of the combined activities measured at the two pH values. Am. J. Med. Genet. 68:173–178, 1997

KEY WORDS: Crigler-Najjar disease types I and II; Gilbert syndrome; bilirubin UDP-glucuronosyltransferase; prenatal diagnosis; hyperbilirubinemia; pH-sensitive activit; jaundice

INTRODUCTION

Although a mildly jaundiced phenotype without significant clinical impact was first described by Gilbert and Lereboullet [1901], it was the description of the severe and inheritable hyperbilirubinemia designated Crigler-Najjar disease [Crigler and Najjar, 1952] that spawned investigations of bilirubin metabolism [Schmid, 1956, 1957]. Hepatic bilirubin UDP-glucuronosyltransferase (transferase) was shown to be responsible for the detoxification of bilirubin through conjugation to glucuronic acid, generating a bilirubin IXαC8-monoglucuronide, a bilirubin IXαC12-monoglucuronide, or a bilirubin IXαC8,C12-diglucuronide. The bilirubin glucuronides have a much higher water solubility than bilirubin and are thus readily excreted from the body, primarily via the biliary system, with a significant circulating fraction secreted in the urine [Kaplan and Isselbacher, 1994].

Defective bilirubin glucuronidating activity was shown to be responsible for three different levels of unconjugated hyperbilirubinemias, presumably related to the deleterious effect of the genetic alteration. The serum bilirubin levels in patients with Gilbert syndrome, affecting some 6% of the population, range between 0.8–3.0 mg % (13.6–51 μM), compared to the normal level of 0.5 mg % (8.5 μM), and levels in individuals with the less prevalent Crigler-Najjar (CN) type II syndrome range between 3.0–20 mg % (51–342 μM). Patients with the rare but lethal CN type I disease have bilirubin levels in excess of 20 mg %, which generally leads to its deposition in the brain (kernicterus) and lethal neurotoxicity. The type I disease is recessively inherited, whereas the pattern of inheritance of type II and Gilbert syndromes has not been clearly defined [Arias et al., 1969; Hunter et al., 1973].

The recent characterization of the *UGT1* gene complex locus [Ritter et al., 1992a] provides a basis for understanding the genetics of the clinical entities. Description of the locus, which utilizes both a series of unique exons with each having 5′ proximal promoter elements and a set of common exons to encode both multiple bilirubin and phenol transferases [Ritter et al.,

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1992a], has led to the identification of four types of mutations in the major bilirubin transferase gene, *UGT1A*. Frameshift [Ritter et al., 1992b; Labrune et al., 1994], nonsense [Bosma et al., 1992; Moghrabi et al., 1993b; Labrune et al., 1994], and missense [Aono et al., 1993; Moghrabi et al., 1993a; Labrune et al., 1994; Erps et al., 1994; Ciotti et al., 1995] mutations are prevalent at this locus. An in-frame codon 170 deletion mutant [Ritter et al., 1993], HUG-Br1-Δ170, encoded within the unique exon 1A with essentially normal activity at pH 7.6, prompted experiments which uncovered 2–4-fold greater activity at pH 6.4 than that at pH 7.6 using human liver microsomes. The HUG-Br1Δ170 mutant that exhibited no detectable activity at pH 6.4 and essentially normal activity at pH 7.6 was considered pH-sensitive. Because the relative amount of messenger RNA coding for the bilirubin-conjugating enzymes, HUG-Br1 and HUG-Br2, is 4:1 [Ritter et al., 1991], it is assumed that HUG-Br1 accounts for at least 80% of the total bilirubin-glucuronidating capacity of the liver.

Certain missense mutations at this locus in CN I individuals are shown to inactivate completely the major bilirubin transferase [Erps et al., 1994; Ciotti et al., 1995] at both pH values. Seppen et al. [1994] demonstrated the presence of partial activity at the typical assay condition of pH 7.6 for the HUG-Br1L175Q mutant. This result agrees with our findings [Ciotti et al., in preparation] on the L175Q mutant, which showed no activity at pH 6.4, but approximately 50% of normal activity at pH 7.6.

Another aspect of bilirubin inactivation is based on the arrangement of the locus. It is predicted that a deleterious mutation in a unique exon 1 inactivates a single isoform, whereas a similar mutation in a common exon (2–5) affects each of the isoforms encoded at the *UGT1* gene complex [Owens and Ritter, 1992, 1995]. The arrangement, no doubt, underlies the observation [Van Es et al., 1990] that some explanted liver specimens of CN I patients have normal phenol transferase activity, while others have greatly reduced activity.

In this report we identified mutations in 3 unrelated CN I patients: a homozygous two-base mutation (P387R) in a common exon at the *UGT1* locus of a 1-year-old, and a heterozygous pattern in both parents and an unborn sibling during the second trimester. In 2 other patients, compound heterozygous mutations, P387R and Q357st, and a homozygous CT deletion in codons 40/41, respectively, were uncovered. Furthermore, we show that the mutated major bilirubin transferase, HUG-Br1P387R, had residual activity at pH 7.6, but was completely inactive at pH 6.4.

MATERIALS AND METHODS

CN Type I Human Samples

B.G. is a male of El Salvadoran descent who presented at age 2 days with unconjugated hyperbilirubinemia. The patient, the first offspring, was hospitalized with an unconjugated hyperbilirubinemia for which he received phototherapy. (Phototherapy changes the 5 and 15 bridges of the *trans* or Z configuration of native bilirubin IXα-ZZ, an internally hydrogen-bounding water-insoluble species, to an E or *cis* configuration with no internal hydrogen-bounding. The resulting ZE/EZ/EE species are more polar and excretable than bilirubin IXα-ZZ.) At age 2 weeks (weight, 3.8 kg), his serum bilirubin of 35.9 mg/dl did not respond to a 10-day trial of phenobarbital treatment administered as an initial load of 15 mg/kg and maintained on 5 mg/kg/day. B.G. underwent orthotopic liver transplantation at age 15 months; the postoperative course was complicated by acute allograft rejection requiring a high-dose methylprednisolone (solumedrol) pulse and a 10-day course of intravenous administration of the murine monoclonal antibody, OKT3, against CD3 cells. Following a third bout of rejection, he was started on tacrolimus (FK506) and is doing well.

The mother became pregnant a second time and underwent amniocentesis at 17.3 weeks of gestation without complications. Cells in the amniotic fluid were pelleted, plated, and grown in cell-culture dishes in Chang medium supplemented with pencillin, streptomycin, and L-glutamine. J.I.G. was born at term via natural, spontaneous vaginal delivery. The newborn was breastfed and showed jaundice with a bilirubin of 11.6 mg/dl on day 5 of life. Her jaundice spontaneously abated, and J.G. has done well without further complications.

Y.A. is a male teenager of Turkish descent; G.D. is a 2-year-old boy of African-American descent.

A clinical research protocol was approved by an institutional review board to study the possible causes of Crigler-Najjar diseases. Informed consent was obtained from all patients participating in this study.

Isolation of Genomic DNA

The sources of reagents used to carry out recombinant DNA techniques have been described [Ritter et al., 1993]. Genomic DNA was isolated from blood samples of all affected individuals (B.G., Y.A., and G.D.), and from the parents of B.G. and Y.A. as previously described [Ritter et al., 1992b]. Genomic DNA was extracted from an amniotic cell culture prepared from the second offspring (J.G.) prenatally, using the standard Qiagen method.

Polymerase Chain Reaction, Subcloning, and Sequencing of PCR Products

In order to localize mutations responsible for inactivating the major bilirubin transferase gene, *UGT1A*, the four common exons were polymerase chain reaction (PCR)-amplified using the sense and antisense primer sets as follows: PXG3 and PXG4 for exon 2, PXG5 and PXG6 for exon 3, PXG7 and PXG8 for exon 4, and PXG9 and PXG10 for exon 5, as described [Ritter et al., 1993]. Exon 1 was amplified as a 990-bp fragment using the sense and antisense primer set PAG4 and PAG5, as described [Ritter et al., 1993]. Each exon was subcloned into pCRII™ vector (Invitrogen, San Diego, CA) and sequenced, using the SP6 and T7 primers in addition to custom-synthesized internal primers for the 990-bp exon-1 insert.

Construction of Wild-Type and Mutant pHUG-Br1 Expression Units

Construction of the pSVL-based expression unit, pHUG-Br1, for synthesis of the normal HUG-Br1 pro-

tein has already been described [Ritter et al., 1991]. The pHUG-Br1P387R unit containing the Pro 387 Arg mutation was constructed in the pSVL-based HUG-Br1 unit. A double mutation was introduced independently by site-directed mutagenesis in both strands, converting CC to GT. Independent PCR reactions were carried out: the S387R sense primer (5′GCAATGGCGTTCGTATGGTGA3′) with the AG2 antisense primer (5′CTGTCTGCACGTCCTCTGAA3′) generated a 617-bp fragment, and the A387R antisense primer (5′TCACCATACGAACGCCATTGC3′) with the P2S4 sense primer (5′CTGTGCGACGTGGTTTA3′) generated a 487-bp fragment. The above fragments were used in a new PCR reaction using P2S4 and AG2 as outside primers to generate a 1,204-bp fragment that was digested with the endonucleases *Afl*III and *Bst*EII and subcloned into *Afl*III/*Bst*EII-digested pSVLHUG-Br1, the wild-type expression unit. The 749-bp fragment contained the codon (CGT) for Arg at position 387. The replaced segment, including the ligation sites of the pHUGBr1P387R construct, was sequenced to ensure that no other changes occurred in the reading frame.

Transfection of pHUG-Br1 and pHUG-Br1P387R Into COS-1 Cells, and Assay for Expression of Protein and Bilirubin Glucuronidation

The wild-type (pHUG-Br1) and the mutant (pHUG-Br1P387R) expression units were transfected [Ciotti et al., 1995; Ritter et al., 1993] into COS-1 cells, and the corresponding wild-type- and mutant [^{35}S]-methionine-labeled bilirubin UDP-glucuronosyltransferases were determined as described [Ritter et al., 1993]. The ^{35}S-methionine-labeled proteins were quantitated in order to normalize wild-type and mutant protein in the glucuronidation assays. The capacity of the transfected cell homogenates to produce bilirubin [^{14}C]-glucuronide was assessed at both pH 6.4 and 7.6, as already described [Ritter et al., 1993; Ciotti et al., 1995]. Because UDP-glucuronosyltransferases contained in the endoplasmic reticulum are latent and require activation/perturbation to allow complete expression of activity when analyzed in vitro, cellular homogenates were detergent-treated with 0.5 mg 3-[(3.-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) per mg protein. (The zwitterion, CHAPS, acts as an efficient perturbant/solubilizing agent without adversely affecting activity over a broad range of detergent-to-protein ratios.) The bilirubin glucuronide product was separated by TLC chromatography and scanned on the Ambis Radioanalytical Imaging System II (San Diego, CA) for quantitation as described [Ritter et al., 1990]. TLC plates were exposed to X-ray film to generate radiograms.

RESULTS

Two-Base Mutation at Codon 387

A two-base substitution in codon 387 (Fig. 1, solid circles) in the common exon 4 of the *UGT1A* gene converted CCC to CGT, replacing a Pro for Arg (P387R) in the carboxyl terminal of HUG-Br1. Sequences of subclones of the other exons were identical to those from a normal individual. Each of five different subclones contained this alteration, establishing the genome as homozygous for the above mutation.

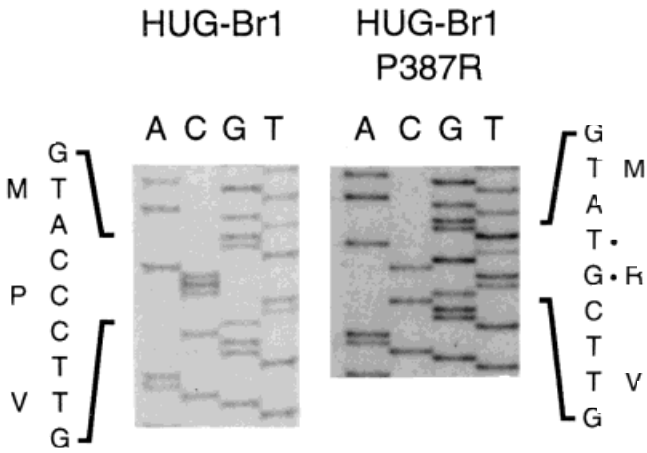

Fig. 1. Autoradiograms of Sanger nucleotide sequencing reactions from a normal and from the CN I *UGT1* gene of patient B.G. Plasmid DNA containing the entire exon 4 from a normal gene (represented by HUG-Br1) and from CN I patient B.G. (represented by HUG-Br1P387R) was sequenced using the pCRII™-specific primers, and sequence ladders were generated by electrophoresis as previously described [Ritter et al., 1992b]. **Lanes A, C, G**, and **T** correspond to separate reactions with added dideoxy derivatives of ATP, CTP, GTP, and TTP, respectively. Solid circles represent the two nucleotides substituted at codon 387 in the genome of the patient.

Two out of six subclones of exon 4 from GD contained the same two-base (GT) mutation, P387R, as observed for BG. Three subclones out of five revealed a C to T transition at codon 357, converting the Gln codon stop codon, TAA, for GD, as shown in Figure 2. The results indicated that GD is a compound heterozygote, P387R and Q357st, for the major bilirubin isoform, HUG-Br1. HUG-Br1Q357st, missing 176 out of 533 amino acids, is expected to be inactive.

All six subclones of exon 1A amplified from the DNA of Y.A. showed a CT deletion for nucleotides 120–121 (codons 40/41), as seen in Figure 3. The homozygous mutation generates a protein of 40 amino acids followed by 14 unrelated amino acids and a premature stop signal at codon 54.

Inheritance of Defective Alleles

Sequence data (not shown) on 5–6 subclones of exon 4 amplified from lymphocyte DNA of each parent of B.G., who claim nonconsanguinity, revealed that each is a carrier for the same mutation. Since a major objective was to establish the genotype for B.G.'s unborn sibling, sequence data were determined on 4–5 subclones of exon 4 derived from DNA isolated from an amniotic cell-culture preparation. The fetus, like both parents, was heterozygous for the P387R mutation (data not shown).

Parental DNA samples of G.D. were not available for analysis. Through DNA sequencing we established that both the mother and the father of Y.A. were carriers of the same CT deletion.

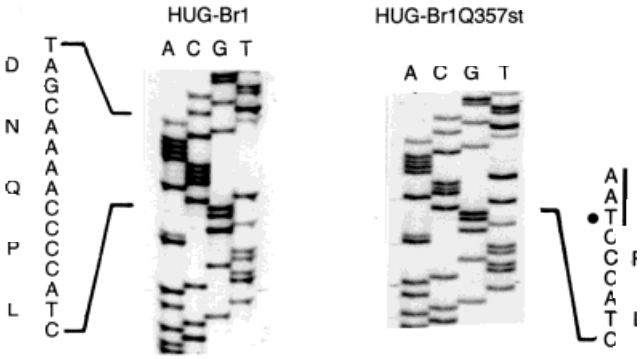

Fig. 2. Autoradiogram of nucleotide-sequencing reaction from a normal and from the CN I *UGT1* gene from patient G.D. Plasmid DNA containing the entire exon 3 from both a normal individual and the proband, G.D., were sequenced and processed as described in Figure 1. Solid circle represents the nucleotide substituted to generate the stop codon, TAA, in one allele of the *UGT1* gene of G.D. The other allele contains the mutation seen in B.G., as described in Figure 1.

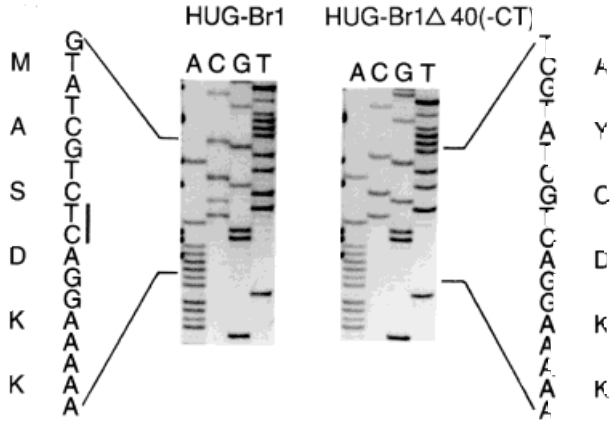

Fig. 3. One of the nucleotide-sequencing reactions from a normal and from the CN I *UGT1* gene of patient Y.A. Plasmid DNA containing the entire exon 1 from both a normal individual and from the CN I gene from Y.A. were sequenced and processed as described in Figures 1 and 2. The dark vertical line denotes the CT that was deleted at codons 40/41 in exon 1A in both alleles of the *UGT1* gene of Y.A.

Expression of [^{35}S]-Methionine-Labeled HUG-Br1 and HUG-Br1P387R Proteins, and Respective Bilirubin Transferase Activities

In order to determine the effect of the P387R mutation on bilirubin glucuronidation, the protein synthesized by pHUG-Br1P387R was analyzed. The data shown in Figure 4 established that both the wild-type and mutant proteins were expressed following transfection of the plasmid units into COS-1 cells. The radioactive band representing the mutant protein is broader than the wild-type band, perhaps due to less efficient glycosylation of the nascent polypeptide. The ^{35}S-methionine label in the proteins was used to quantitate and normalize the amount of wild-type and mutant proteins used in the glucuronidation assays to account for this difference in expression. The quantitation of the bilirubin [^{14}C]-glucuronide products generated by the two expression units, and separation by TLC chromatography, showed that the wild-type HUG-Br1 protein generated 4-fold (484 $\pm$ 72 counts) more product at pH 6.4 than at pH 7.6 (126 $\pm$ 17 counts), as seen in Figure 5. The mutant protein formed essentially no product at pH 6.4, but did generate 26% (33 $\pm$ 25 counts) of the normal level at pH 7.6. This residual activity was 5.4% of the total product. All activities were determined on samples from three independent experiments, following storage of cells at -70°C for several days, as described in Figure 5. The residual activity of the mutant protein consistently generated product at pH 7.6. Control COS-1 cells generated no product at either pH value. Radiograms of the product were taken from exposures of the TLC plates to X-ray film.

DISCUSSION

Characterization of the human bilirubin transferase cDNAs [Ritter et al., 1991] and the subsequent description of the corresponding genetic locus *UGT1* [Ritter et al., 1992a] have been critical in determining the genetic basis of bilirubin transferase deficiencies. In this study we used this technology for the first time to establish, before birth, that a fetus at-risk for the critical CN I disease is a carrier of a deleterious mutation. Since there are some 33 different mutations among some 44 patients examined to date and reported in the literature (see Introduction), almost each individual has a different genotype. Hence, we established: 1) the location of the mutations from the affected offspring, and 2) the effect of the mutation, P387R, on HUG-Br1 activity. Thus, this information would allow us to make a definitive assertion concerning the deleterious nature of the mutation in the genome of the at-risk fetus. This assertion assumes that the activity of the bilirubin enzyme in the endoplasmic reticulum of the COS-1 kidney cell does not differ substantially from that in hepatocytes.

The mutant HUG-Br1P387R enzyme with Arg replacing the conserved Pro 387 is completely inactive at major pH 6.4, but retains minimal activity at minor pH 7.6. The importance of establishing the effect of the mutation was demonstrated in a previous study [Ciotti et al., 1995], where the substitution of conserved Pro 285 had no effect on bilirubin glucuronidation by the HUG-Br1 protein. Further, the HUG-Br1L175Q mutant contained residual activity in the traditional pH-7.6 assay conditions [Seppen et al., 1994]. With the L175Q mutant protein, we also found activity (50%) at pH 7.6, but total lack of activity at pH 6.4 (Ciotti and Owens, in preparation). This and our previous study [Ritter et al., 1993], showing a loss of activity at major pH 6.4, provides a rationale for the severe CN I phenotype despite the presence of 26% normal activity at minor pH 7.6. This residual activity (6% of total) is, no doubt, insufficient to reduce significantly the hyperbilirubinemic status of the patients. The report by Kaufman et al. [1986] on patient G.R., with the HUG-Br1Δ170 mutation, indicated that detectable quantities of bilirubin conjugates (12.9%) were consistently found in the duodenal and

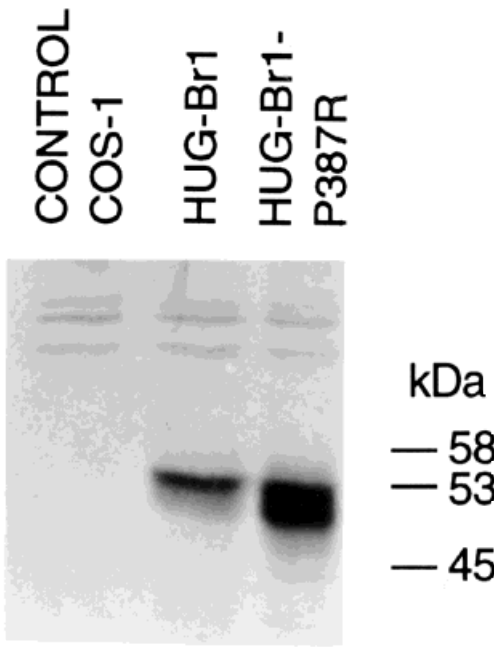

Fig. 4. Immunocomplexes of pHUG-Br1- and pHUG-Br1P387R-transfected cells compared to COS-1 control cells. Cells were transfected with the normal pHUG-Br1 or the mutant pHUG-BR1P387R expression unit, radiolabeled with [^{35}S]methionine, solubilized, and immunocomplexed with goat antimouse UDP-glucuronosyltransferase IgG as described [Ritter et al., 1993; Ciotti et al., 1995]. The radiolabeled proteins were run on a 7.5% SDS polyacrylamide gel and processed for autoradiography as described earlier [Ritter et al., 1990].

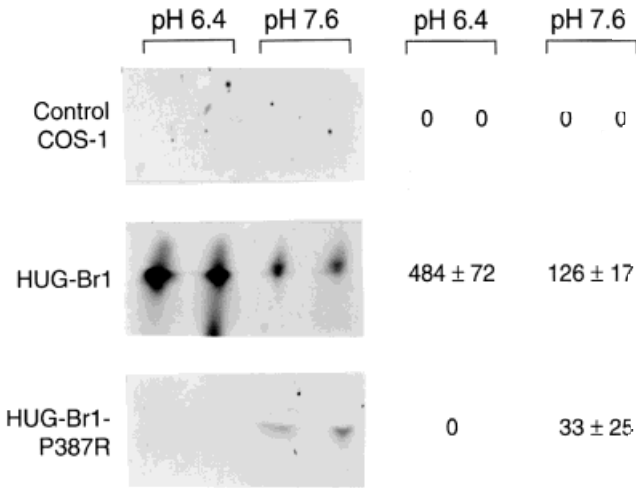

Fig. 5. Bilirubin [^{14}C]glucuronide generated by the normal HUG-Br1- or the mutant HUG-Br1P387R-enzyme. Homogenates of COS-1 cells transfected with pHUG-Br1 or pHUG-Br1P387R were assayed at the major pH optimum (6.4) and at the minor pH optimum (7.6) according to published procedures [Bansal and Gessner, 1980; Ritter et al., 1993]. The glucuronidation reaction (1.41 mM [^{14}C]UDP-glucuronic acid at 1.4 μCi/μM) contained equivalent specific protein determined by ^{35}S-methionine-labeled proteins, and continued for 16 hr at room temperature. Cellular protein was detergent-treated with 0.5 mg CHAPS/mg protein. The protein levels for HUG-Br1 and HUG-Br1P387R were 1.2 mg and 0.3 mg, respectively. The counts in bilirubin-β-[^{14}C]glucuronides represent the average ± standard error (n = 8) generated in three experiments. The product was separated by TLC chromatography, and the TLC plates were exposed to X-ray film which was printed for a radiogram [Ritter et al., 1993]. The region of the print reflecting the product is shown.

gallbladder bile, and suggested that minute amounts of bilirubin-glucuronidating activity might exist. Thus, HUG-Br1P387R represents the third mutant, including HUG-Br1Δ170 and HUG-Br1L175Q, found in CN I patients with residual activity at pH 7.6.

Although it is not known how Pro 387 is critical to the structure and activity of the HUG-Br1 enzyme, its location in the common end of the proteins as defined by the *UGT1* locus suggests that it more directly influences the site of interaction of the common donor substrate, UDP-glucuronic acid, than that of the bilirubin acceptor substrate. A report by Mackenzie [1990] showed that the first 297 amino acids in the NH_2 terminal portion of two UDP-glucuronosyltransferases determine substrate selection. This observation supports the finding that any unique exon at the *UGT1* locus encoding approximately 289 amino acids is sufficient to determine acceptor substrate selection.

In the cases of three other missense mutants [Erps et al., 1994; Ciotti et al., 1995], bilirubin transferase activity was completely abolished at both pH values. Furthermore, the use of both the genetic and the biochemical data for the P387R mutant would permit us to make a definitive determination of the potential risk to the unborn fetus.

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